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METABOLISM OF TYROSINE TO *p*-HYDROXYPHENYL-PROPIONIC ACID AND TO *p*-HYDROXYPHENYLACETIC ACID BY THE HAEMOLYMPH OF THE AMERICAN COCKROACH

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ABSTRACT

Labelled tyrosine (^{14}C-U) is metabolized through two different pathways: tyrosine → *p*-hydroxyphenylpyruvic acid → *p*-hydroxyphenylacetic acid and tyrosine → *p*-hydroxyphenylpyruvic acid → *p*-hydroxyphenyl-lactic acid → *p*-hydroxyphenylpropionic acid.

These reactions have been shown to take place *in vitro* using partially purified enzymes from whole haemolymph. The purification procedure involved column chromatography on Sepharose 6B which effectively eliminated tyrosinase-phenoloxidase participation via removal of necessary cofactors and/or activators.

Tyrosine decarboxylase is in competition for the substrate since pyridoxal phosphate is an essential cofactor for both this enzyme and the synthesis of the four acids. Subsequent β-hydroxylation of tyramine can be inhibited by the deletion of ascorbic acid from the reaction mixture.

The synthesis of the acids can be obtained by using haemolymph from newly ecdysed larvae, intra-ecdysal larvae, and adult female cockroaches. Thus the enzyme system is present throughout the life of the insect and the synthesis is not 'switched off' during ecdysis as it is in blowflies.

TYROSINE metabolism in insects has been extensively studied during the preceding decades (reviews by Brunet, 1963, 1965; Cottrell, 1964; Hackman, 1964). In the American cockroach tyrosine can be hydroxylated to dopa or decarboxylated to tyramine (Mills, Lake, and Alworth, 1967) and both of these metabolites can be converted to dopamine (Whitehead, 1969). However, a third major metabolite, *p*-hydroxyphenyl-pyruvic acid, arises from tyrosine via transamination or deamination (Mills and others, 1967) but its metabolism in the cockroach has not yet been studied.

In blowflies, Karlson and Sekeris (1962) have shown that *p*-hydroxyphenylpyruvate is converted to *p*-hydroxyphenylpropionate with the lactate as an intermediate. Apparently this pathway is operational only in the interecdysial period and tyrosine metabolism is switched towards the synthesis of *N*-acetyldopamine by the induction of dopa decarboxylase. In our preliminary studies with the American cockroach, it was found that this pathway functions throughout the life of the animal. *In vitro* blood preparations from newly ecdysed larvae and adults cause the rapid metabolism of tyrosine to various acidic compounds. The present work describes the identification of a number of these and discusses their possible role in cuticle and oothecal sclerotization.

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METHODS

Experimental Animals

American cockroaches [*Periplaneta americana*] were used in all experiments and were held in large rearing aquaria containing food and water at all times. A constant temperature of 25° C. was maintained and the photoperiod was 12 hours light, 12 hours dark. Newly ecdysed larvae (sixth to last instar) were removed as needed and held in glass jars under the same conditions as above. At various times between ecdysis (26–30-day duration) animals were removed. Adult female cockroaches were 20–45 days old and were producing an ootheca every 5–6 days. Haemolymph obtained at any time during the cycle was enzymatically active.

Enzyme Preparation and Assay

Two parts of whole blood were diluted with 1 part distilled deionized water, finely ground in a ground glass homogenizer, and centrifuged at 10,000 *g* for 10 minutes. This was denoted the crude blood homogenate. In subsequent experiments, the supernatant was chromatographed on a 1 × 45 or 1 × 60-cm. column of Sepharose 6B. Elution was with 0·1 *M* potassium phosphate buffer (*p*H 7·6), 1-ml. fractions were collected, and protein was determined by a continuously monitoring U.V. attachment. P-2 polyacrylamide gel was used to concentrate the combined fractions. The Sepharose 6B was prepared by mixing the prepared solution with 3 times its volume of 0·1 *M* potassium phosphate buffer (*p*H 7·6) and degassing it under vacuum for 2 hours. Columns were washed thoroughly with the same buffer before use.

To determine acid formation, 3 µc. UL-^{14}C-tyrosine (16 µl.), 200 µl. enzyme preparation, 10 µmoles pyridoxal phosphate, 5 µmoles NADH + H^+, and sufficient potassium phosphate buffer 0·1 *M p*H 7·6 to make a total volume of 800 µl were incubated at 37° C. for 12 hours. The reaction was stopped by the addition of equal volumes of methanol, and denatured protein was removed by centrifugation.

Identification of the Reaction Products

High-voltage electrophoresis (HVE) and conventional one-dimensional, descending paper chromatography were used to separate the various reaction products. HVE was conducted at *p*H 3·5 pyridine/acetic acid/water, 1:10:89 (4 kV. for 40 minutes). Solvents for paper chromatography were: ethanol/ammonia/water, 16:1:3 (EtAM), *n*-butanol/acetic acid/water, 4:1:1 (BAW), benzene/acetic acid/water, 125:72:3 (BzAW), and isopropanol/ammonia/water, 20:1:2 (iPAW). The acetic acid was glacial and the ammonium hydroxide had a specific gravity of 0·88. Detection was by diazotized sulphanilic acid, $FeCl_3$, $AgNO_3$, and U.V. Radioactive metabolites were resolved with a Tracerlab or Packard strip-counter. Conclusive identification was achieved by cutting out the region containing the compound in question from the electropherogram and re-chromatographing it in at least two other systems.

RESULTS

Tyrosine can be metabolized to *N*-acetyldopamine by the American cockroach with dopa and dopamine being intermediates (Mills and others, 1967). An alternative pathway involving tyramine to dopamine also exists and may be the more important route (Whitehead, 1969). During the course of these studies a number of acidic substances have been found which are derived from tyrosine. One of these has been positively identified as *p*-hydroxyphenylpyruvate and it is feasible to believe that this compound may be further metabolized as shown by Karlson and Sekeris (1962). The initial experiments were designed to study this possibility.

Metabolism by Crude Blood Homogenates

Crude haemolymph from newly ecdysed larvae, having been subjected to osmotic shock (distilled water) and broken up in a ground glass homogenizer, was used as the enzyme source. Incubation of ^{14}C-tyrosine(U) with this preparation resulted in the formation of tyramine, dopamine, and *N*-acetyldopamine which confirmed previous

work. However, high-voltage electrophoresis (HVE) of the reaction products revealed that acidic compounds were also present. As shown in *Fig.* 1, three peaks of radioactivity are present. Peak 1 contains unreacted tyrosine and small amounts of *N*-acetyldopamine. Peak 2 is *p*-hydroxyphenylacetic acid (*p*HPA) while peak 3 is *p*-hydroxyphenyl-lactic acid (*p*HPL). These preliminary experiments suggested that *p*-hydroxyphenylpyruvic acid (*p*HPP) is indeed being metabolized by cockroach haemolymph, but competition for the substrate prevents excessive build-up of the acids. To eliminate this obstacle, the centrifuged crude homogenate was subjected to gel filtration on Sepharose 6B. The large-porosity gel was used initially to remove the large molecular weight phenol oxidase which

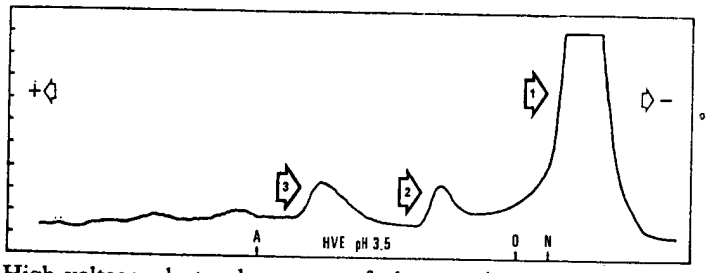

FIG. 1.—High-voltage electropherogram of the reaction products after using crude blood homogenates as the enzyme source. The buffer was pyridine/acetic acid/water 1:10:89 (*p*H 3·5). N is the neutral point, O the origin, and A the aspartic acid reference. Electrophoresis was conducted at 4 kV. for 40 minutes. Peak 1 is unreacted tyrosine, peak 2 is *p*-hydroxyphenylacetic acid, and peak 3 is *p*-hydroxyphenyl-lactic acid.

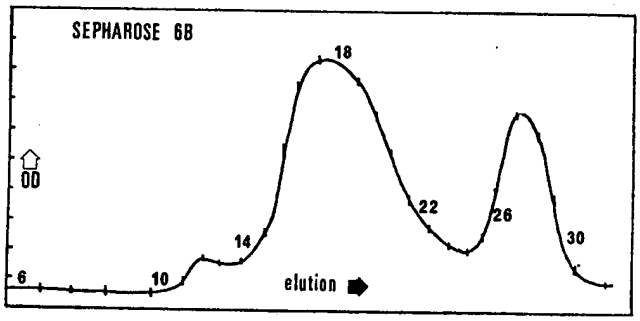

FIG. 2.—Chromatography of the soluble blood proteins on a 1×60-cm. column of Sepharose 6B. Elution was with 0·5 *M* phosphate buffer (*p*H 7·4) and 1-ml. fractions were collected.

is known to be present in haemolymph (Mills, Androuny, and Fox, 1968). This was not wholly achieved but hydroxylation was eliminated, supposedly by the removal of cofactors and/or activators.

TYROSINE METABOLISM WITHOUT TYROSINE HYDROXYLASE

The soluble haemolymph preparation eluted off a 1×60-cm. column of Sepharose 6B separated into three distinct peaks (*Fig.* 2). Tubes 17, 18, 19 from the second peak corresponded to the correct molecular weight for most enzymes (100,000–350,000) so these were combined and concentrated to one-third their original volume with P-2 polyacrylamide gel.

This enzyme source, when incubated with ^{14}C-tyrosine(U), catalysed the formation of some additional acidic substances (*Fig.* 3). It should be noted that ascorbic acid was added as a precaution against oxidation at this time. The numbered peaks are as follows: (1) unmetabolized tyrosine, *p*-hydroxyphenylpropionic acid (*p*HPPn), and a trace of *p*-hydroxybenzoic acid (*p*HB); (2) *p*HPA; (3) *p*HPL; (4) *p*-hydroxymandelic acid (pHM), and (5) *p*HPP. These results confirmed the presence of the pyruvate (pHPP) and presented evidence that further metabolism to the acetate (*p*HPA) and to the propionate (*p*HPPn) via the lactate (*p*HPL) occurred. The detection of the mandelate (*p*HM) was surprising since this would initially involve the β-hydroxylation of tyramine

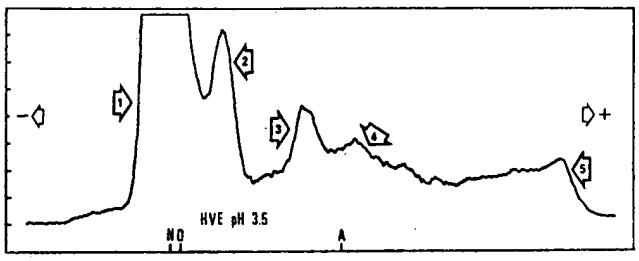

FIG. 3.—High-voltage electrophoresis of the reaction products after removing the influence of tyrosine hydroxylase. The enzyme was the partially purified fraction obtained from the column of Sepharose 6B (tubes 17–19 in *Fig.* 2). Electrophoresis was as in *Fig.* 1. The identification of the numbered peaks is as follows: 1, Tyrosine, *p*HPPn, *p*HB; 2, *p*HPA; 3, *p*HPL; 4, *p*HM; 5, *p*HPP.

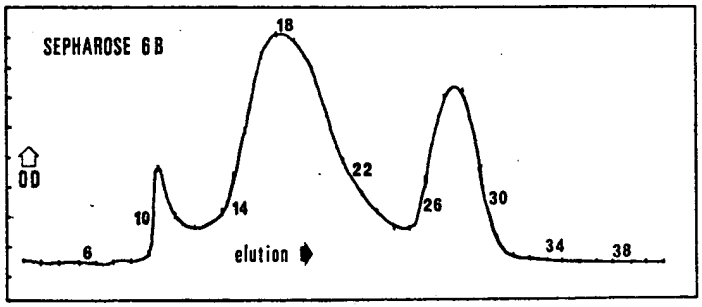

FIG. 4.—Chromatography of the soluble blood proteins on a 1 × 70-cm. column of Sepharose 6B. Elution was as in *Fig.* 2.

to octopamine (Lake, Mills, and Brunet, 1971) and its subsequent conversion to mandelate via monoamine oxidase (Mills, Lake, and Brunet, 1971). Since the β-hydroxylase should be in the microsomal fraction contained in the first peak (*Fig.* 2) it was surmised that either the hydroxylase had been solubilized or one or more of the four acids (pyruvate, lactate, propionate, or acetate) was being converted to the mandelate. In order to answer this question, a slightly longer column with a reduced flow rate was employed to separate the haemolymph preparation.

TYROSINE METABOLISM WITHOUT β-HYDROXYLATION

The slight modification in the column failed to achieve any better separation (*Fig.* 4). However, the separation attained was sufficient so that both the first peak (tube 12) and

the second peak (16, 17) were concentrated as before when incubated with ^{14}C-tyrosine (U). From *Fig.* 5 it can be seen that the first peak (*Fig.* 4) which contained the larger proteins was able to catalyse the metabolism of tyrosine to a large variety of acids. Identification of the numbered peaks is included in the figure. Thus, the enzymes capable of converting tyrosine to *p*HB are present in the large protein fraction which may contain membrane fractions from the mitochondria and other organelles.

The second incubation utilized tubes 16–17 from the second peak and one group contained ascorbic acid while the other did not. *Fig.* 6 shows the reaction products after

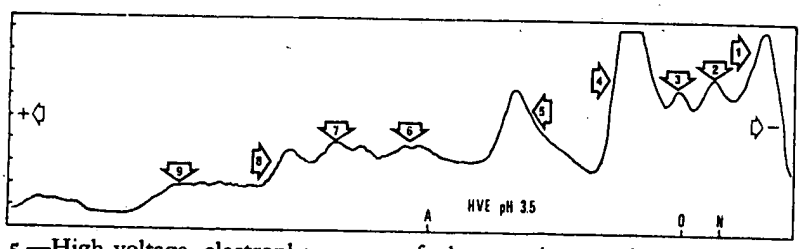

FIG. 5.—High-voltage electropherogram of the reaction products after using high molecular weight substances as the enzyme source. The enzyme source was tube 12 from the elution curve shown in *Fig.* 4. HVE was as in *Fig.* 1. The numbered peaks were identified as: 1, Tyrosine; 2, *p*-Hydroxybenzaldehyde; 3, *p*HB; *p*HPPn; 4, *p*HPA; 5, *p*HPL; 6, *p*HM; 7 and 8, unknown; 9, *p*HPP.

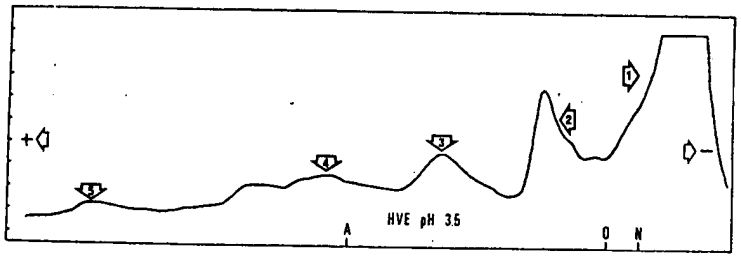

FIG. 6.—High-voltage electropherogram of the reaction products after using proteins of approximately 150,000 molecular weight as the enzyme source. The enzyme source was the concentrate of fractions 16–17 from the elution pattern shown in *Fig.* 4. HVE was conducted as in *Fig.* 1. The numbered peaks are identified as: 1, Tyrosine, *p*HPPn; 2, *p*HPA; 3, *p*HPL; 4, *p*HM; 5, *p*HPP.

HVE when ascorbic acid was added. However, if ascorbic acid was deleted, the electropherogram was essentially the same except that peak 4 containing the *p*-hydroxymandelic acid was absent. These data indicate that small amounts of the β-hydroxylase have become solubilized and are contained in the smaller protein fraction. However, the deletion of ascorbate effectively removes its participation and suggests that under the conditions used, mandelate is not derived from the four acids (*p*HPP, *p*HPL, *p*HPPn, or *p*HPA).

TYROSINE CONVERSION TO THE PHENOLIC ACIDS DURING VARIOUS STAGES OF THE LIFE CYCLE

Immature cockroaches in the intra-ecdysal period were able to metabolize tyrosine to the four phenolic acids at a rapid rate. The relative age within the period was not a factor since animals 2, 6, 10, 14, 18, and 22 days after the previous ecdysis had approximately

the same specific activity. However, the total number of cells (and total blood protein) increased prior to apolysis which caused an elevation in absolute enzyme levels. This observation led to the analysis of haemolymph from cockroaches in the process of ecdysis.

When blood from newly ecdysed insects is incubated with ^{14}C-tyrosine the four acids are again synthesized at a rapid rate. In addition, crude blood also produces the acids which indicate that their synthesis may occur *in vivo*. Further experiments showed that adult female blood was highly active during the entire 5–6-day vitellogenic cycle.

DISCUSSION

The results presented in this communication prove that haemolymph from the American cockroach is capable of metabolizing tyrosine to *p*-hydroxyphenylpyruvic acid, β-hydroxyphenyl-lactic acid, *p*-hydroxyphenylpropionic acid, and *p*-hydroxyphenylacetic acid. The probable sequence of reactions is outlined in *Fig.* 7.

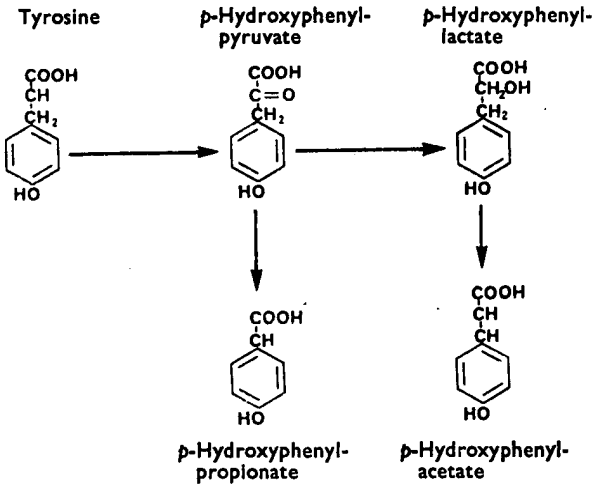

FIG. 7.—Pathways of tyrosine metabolism.

The same blood-cells contain a β-hydroxylase which is primarily associated with the microsomal fraction. Some soluble hydroxylase is also present but fails to function when ascorbic acid is omitted from the reaction mixture. From these data it was determined that *p*-hydroxymandelic acid was not derived from any of the four acids but arose as a result of β-hydroxylation.

Haemolymph obtained from freshly ecdysed larvae, interecdysyl larvae, and from adult females all metabolized tyrosine to the four acids at an approximately equal rate. Thus, the cockroach is different from the blowfly (Karlson and Sekeris, 1962) in that this particular pathway is not confined to a non-developmental stage but functions throughout the life of the insect. The postulate that this pathway may be degradative in nature cannot be ruled out. However, the rather abundant *in vitro* synthesis of these acidic substances would tend to support the contention of Brunet (1965) that they are converted into protocatechuric acid by some as yet undefined pathway.

Recent evidence that other phenolic compounds may be incorporated into the insect cuticle (Anderson, 1970; Koeppe and Mills, 1970) suggests that perhaps these phenolic

acids may function as cofactors or auto-oxidizers during sclerotization. The fact that they are apparently synthesized *in vivo* and that little ^{14}C-tyrosine metabolite is ever detectable in the faeces immediately after ecdysis (Mills, 1970) would indicate that the four phenolic acids are utilized in some way. Their synthesis during the production of an ootheca may be due to their possible metabolism to protocatechuic glucoside (Brunet, 1965). Whitehead (1969) has obtained evidence that the acetaldehyde can be converted to protocatechuic glucoside *in vivo*.

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Key Word Index: Tyrosine metabolism, cockroach haemolymph, *p*-hydroxyphenylpropionic acid, *p*-hydroxyphenylacetic acid.